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Regulation of Solute Transport in Streptococci by External and Internal pH Values

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INTRODUCTION

Solute transport systems in lactic acid bacteria can be classified into three categories according to the mode of energy coupling (for a recent review, see reference 28): (i) secondary transport systems which utilize electrochemical energy; (ii) adenosine 5'-triphosphate (ATP)-dependent transport systems driven by chemical (phosphate-bond) energy; (iii) group translocation systems which couple the translocation to a chemical modification of the solute. For some transport systems in lactic acid streptococci, a role of both ATP and the electrochemical gradient of protons (proton motive force [Δp]) has been implicated in either energization or regulation of transport (5, 28).

The mode of energy coupling to solute transport is often deduced from the dependence of the rate of transport on the proton motive force or one of its components or from its dependence on other energy-rich (metabolite) intermediates (13, 20, 27). Often, flow-force relationships are found (Fig. 1A). For many secondary transport systems, a quadratic relationship is observed between the rate of transport and the applied driving force (11, 39). Information about the driving force for secondary transport systems can also be obtained from the relation between the steady-state accumulation level of a solute and the magnitude of the components of the proton motive force (Fig. 1B) (1, 2, 16, 37). At steady state the rate of influx equals the rate of efflux. When both processes are mediated only by the secondary transport carrier, the driving force for net solute uptake at steady state is zero. This driving force is supplied by the solute gradient ($\Delta\bar{\mu}_A/F$) together with the total proton motive force (Δp) across the cytoplasmic membrane or some combination of its components (the electrical potential [$\Delta\psi$] or the chemical proton gradient [$-Z\Delta pH$] or both). From an analysis of $\Delta\bar{\mu}_A/F$, $\Delta\psi$, $-Z\Delta pH$, and Δp under different steady-state conditions, the role of each of these parameters in the driving force can be deduced. Such analyses usually involve modulation of the composition of the proton motive force by specific ionophores. It should be realized that changes in the magnitude or composition of the proton motive force affect more than the driving force for secondary transport. These changes can also result in variations of the internal pH, a parameter which can influence the kinetic properties of the transport system. The effects of the internal pH and also the external pH on secondary transport systems must be properly analyzed to avoid erroneous conclusions about the roles of the $\Delta\psi$ and ΔpH as a driving force for solute transport. On the other hand, an apparent correlation between the rate of solute transport and the magnitude of the ΔpH can be misleading.

The purpose of this paper is to review the various regulatory effects of internal and external pH values on solute transport which can occur in addition to or independent of the role of a pH gradient (ΔpH) in driving solute transport. These regulatory effects are illustrated by studies on proton motive force and phosphate bond-driven transport systems in *Streptococcus lactis*, *Streptococcus cremoris*, and *Streptococcus faecalis*. The effects exerted by the external and internal pHs are discussed separately. Further, a distinction is made between the type of interaction of protons with the transport proteins.

(i) Catalytic pH effects. Carriers which mediate proton-coupled solute transport have at least one binding site for the solute and one binding site for the proton. During the transport cycle these binding sites will be exposed to the outer or inner surface of the membrane or to both. For each solute taken up, the proton binding site has to be protonated at the outer surface and deprotonated at the inner surface of the membrane. Depending on the dissociation constants of these reactions, major changes in the catalytic activity of the carrier occur upon changes of the internal or external pH or both.

(ii) Allosteric pH effects. Carriers may contain regulatory domains which, upon interaction with protons from the water phase, alter the catalytic activity. These allosteric pH

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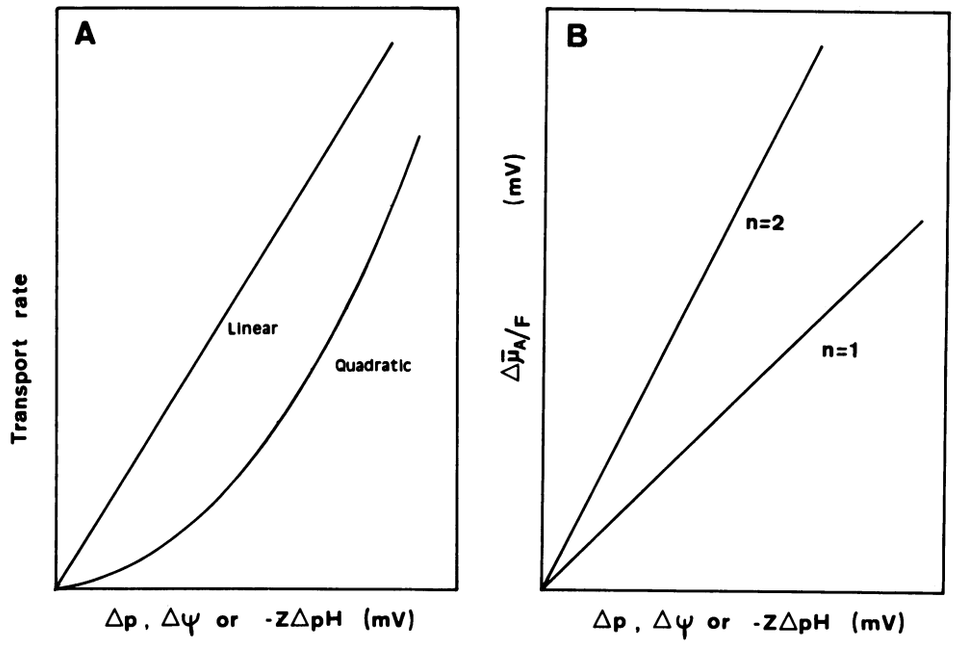

FIG. 1. Effects of proton motive force or one of its components on rate of solute transport (A) and the steady-state level of substrate accumulation (B). Lines are drawn on the assumption that the solute translocated is neutral. n, Number of protons accompanying translocation of the solute.

effects can occur irrespective of the involvement of proton translocation in the translocation cycle.

The physiological consequences of the observed regulatory pH effects on solute transport systems are discussed in a separate section.

INTRACELLULAR pH OF GLYCOLYZING AND GROWING STREPTOCOCCI

Before we describe the various effects of pH on solute transport, we discuss the mechanism of pH regulation in lactic acid bacteria and the methods of manipulation of the intracellular pH. For extensive reviews on bacterial pH homeostasis, the reader is referred to the review by Booth (8).

Glycolyzing cells of *S. lactis* and *S. cremoris* maintain an intracellular pH relatively constant at around 7.5 between external pH values of 5.0 to 7.5 (Fig. 2). Above pH 7.5, the intracellular pH increases almost proportionally with the extracellular pH. *S. lactis* cells metabolizing arginine as a source of ATP synthesis between pH 5.0 and 7.0 maintain an intracellular pH that is 0.3 to 0.4 pH unit lower than in glycolyzing cells at the same external pH.

Provided the concentrations of weak acids (e.g., acetate and lactate) and sodium ions are kept below 50 mM, growing streptococci are able to maintain the intracellular pH at values similar to those observed for glycolyzing cells (Fig. 2). The intracellular pH of chemostat-grown *S. cremoris* Wg2 remains between 6.9 and 7.5 when the extracellular pH is varied from pH 5.5 to 7.5 (41).

The mechanism of pH homeostasis in streptococci has been relatively well studied in *S. faecalis* (23–26). The features of this mechanism are as follows. (i) The extrusion of protons by the F_0F_1 adenosine triphosphatase and the electrogenic uptake of potassium ions raise the cytoplasmic pH. (ii) The activity of the F_0F_1 adenosine triphosphatase decreases with increasing pH. (iii) The synthesis of the F_0F_1 adenosine triphosphatase is regulated by the internal pH. (iv) No mechanism to acidify the cytoplasm exists other than proton release during glycolysis. (v) The net formation of either acidic or basic end products by cells metabolizing lactose and arginine, respectively, does not seem to be essential for pH homeostasis at alkaline pH values, because the intracellular pH becomes even more acid relative to the outside medium with arginine than with a glycolytic substrate (24, 34).

The inability of *S. lactis* and *S. cremoris* cells to regulate

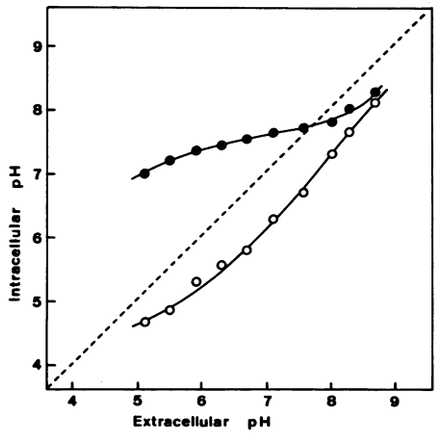

FIG. 2. Cytoplasmic pH of *S. lactis* ML3 in the presence (○) and absence (●) of nigericin (0.8 nmol/mg of protein). Cells were allowed to glycolyze in the presence of lactose for 5 min at 30°C, after which the cells were separated from the medium by silicon oil centrifugation (34). The intracellular pH was calculated from the distribution of either [^{14}C]benzoic acid or [^{14}C]methylamine.

the intracellular pH in alkaline media (Fig. 2) and the absolute requirement for potassium ions to alkalinize the cytoplasm (34) suggest that the mechanism of pH regulation in these organisms could be similar to that in *S. faecalis* (23, 24, 26). Differences in pH homeostasis between *S. lactis* and *S. faecalis* are found with respect to the absolute value at which the internal pH is regulated and the regulation of the internal pH at acidic pH values (24, 34, 36).

To investigate the role of the internal pH on transport processes in *S. lactis* and *S. cremoris*, the cytoplasmic pH can be altered in various ways. Methods that have frequently been used to lower the internal pH include titration with weak acids or with the ionophore nigericin, which catalyzes the electroneutral exchange of potassium ions for protons (2, 6, 34). Alternatively, the internal pH can be manipulated indirectly by the potassium concentration in the medium in the presence of the potassium ionophore valinomycin (31, 34). These methods, however, have the limitation that the changes in the internal pH depend on the magnitude of the pH across the cytoplasmic membrane. By varying the external pH in the presence of a protonophore or the ionophore nigericin, the intracellular pH can be set at any desired value. This internal pH value is not necessarily equal to the external pH. When *S. lactis* cells are incubated with an excess of the ionophore nigericin in the pH range 5 to 9, the pH value of the cytoplasm is 0.7 to 0.8 pH unit lower than the external pH independent of the energy source (Fig. 2). The reversed pH gradient is also observed in the absence of metabolic energy at alkaline pH values or when the intracellular potassium concentration is low. This pH gradient is therefore most likely the result of a Donnan potential.

A transient pH gradient can be generated by diluting cells loaded with a weak acid or a weak base into media containing an impermeant counterion (28). The passive influx of these solutes can also be used to change the cytoplasmic pH. Alternatively, a ΔpH can be created by a sudden shift in the external pH (29). The imposition of these artificial ion diffusion potentials has the disadvantage of the continuous change of intracellular pH with time, which complicates the estimation of the pH gradient.

The methodology for manipulating the internal pH as described above is also applicable to the study of solute transport in membrane vesicles. Although cytoplasmic membrane vesicles of streptococci do not contain a functional proton pump to generate a proton motive force, such a system can be incorporated by a simple reconstitution method (10, 11; see below).

ELECTROCHEMICAL GRADIENT-DRIVEN SOLUTE TRANSPORT SYSTEMS

The translocation of most amino acids across the cytoplasmic membrane of streptococci is driven by the proton motive force (Δp) or one of its components. In *S. cremoris*, distinct and specific transport systems have been recognized for the following structurally related amino acids: L-leucine, L-isoleucine, and L-valine (9); L-serine and L-threonine; L-alanine and L-glycine (A. J. M. Driessen, Ph.D. thesis, University of Groningen, Haren, The Netherlands, 1987). The mechanisms of transport of these amino acids have been studied in detail, using a novel system in which beef heart cytochrome *c* oxidase is incorporated into membrane vesicles of *S. cremoris* to function as a Δp-generating system (10, 11). Membrane vesicles of *S. cremoris* have been fused with proteoliposomes containing cytochrome *c* oxidase by a freeze-thaw/sonication procedure. In the presence of the electron donor system ascorbate/*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine/cytochrome *c*, a constant Δp of up to −120 mV (inside negative and alkaline) can be sustained in these membranes for a long period of time. With this model system, it has been demonstrated that neutral and branched-chain amino acids are translocated in symport with one proton (11a, 12). For uncharged amino acids, cotranslocated with protons, both the ΔpH and Δψ component of the Δp contribute to the driving force for uptake. Unlike the phosphate bond-driven transport systems (see below), protons are one of the substrates for the carrier. The internal and external pHs may therefore exert their effects on both catalytic and allosteric H^+-binding sites.

The effects of pH on facilitated diffusion and Δp-driven transport of L-leucine, L-alanine, and L-serine have been studied extensively (11, 11a, 12). These studies have revealed a remarkable asymmetry with respect to the influence of pH on transport of these amino acids at both sides of the membrane.

External pH Effects

Two different effects of the external pH on the branched-chain amino acid carrier have been recognized, both of which can be considered catalytic pH effects on the carrier function. Studies have been performed with L-leucine as the transported solute. The binding and release of H^+ and L-leucine to and from the carrier occur most likely via an ordered mechanism (9). A complete translocation cycle of carrier-mediated influx proceeds via the following sequential steps (Fig. 3): (i) H^+ binds to the negatively charged carrier on the outer surface of the membrane; (ii) L-leucine binds to the carrier; (iii) transmembrane translocation takes place in which the uncharged carrier-H^+-leucine complex reorients

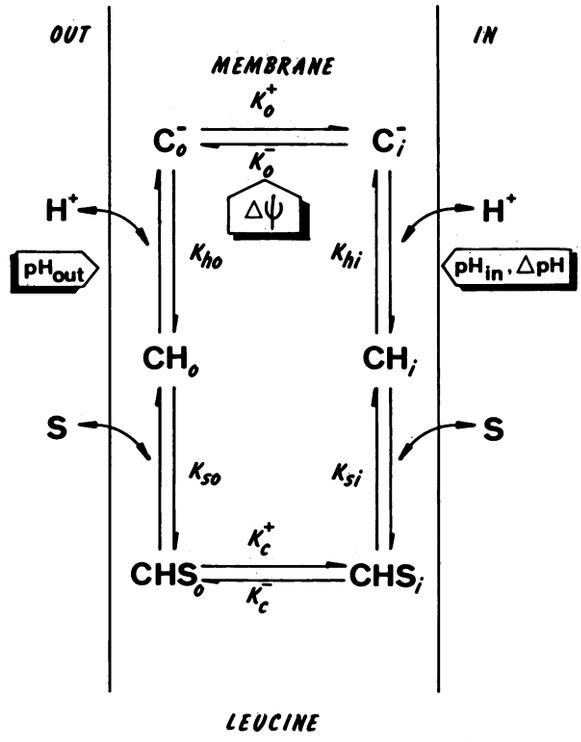

FIG. 3. Symmetrical model for the transport cycle by a carrier (C) catalyzing H^+/L-leucine (S) cotransport.

from the outer to the inner surface of the membrane and (iv) L-leucine is released on the inner surface of the membrane; (v) H^+ is released on the inner surface; and (vi) finally, the unloaded negatively charged carrier reorients from the inner to the outer surface of the membrane.

The effect of the external pH on the kinetics of leucine uptake was studied. The K_t for L-leucine uptake was found to exhibit a marked external pH dependency (Fig. 4). A sigmoidal increase in affinity (decrease in K_t) was observed with increasing external H^+ concentration, which most likely implies a positive cooperativity between H^+ and L-leucine binding. The apparent pK (pK_a) for this process is about 7.0. Importantly, the change in K_t as a function of pH is independent of the nature of the driving force, e.g., imposed $\Delta\psi$ (Fig. 4, open circles) or imposed ΔpH (Fig. 4, closed circles). A similar change of K_t as a function of the external pH was observed when the driving force for L-leucine was supplied by the action of cytochrome *c* oxidase. These observations demonstrate that H^+ binding to the carrier on the external surface of the membrane increases the affinity of the carrier for L-leucine.

A second external pH effect was observed when L-leucine efflux was assayed. During L-leucine efflux, the translocation cycle described above proceeds in the reversed order. L-Leucine efflux from membrane vesicles of *S. cremoris* occurs with pseudo-first-order rate kinetics (Fig. 5A). The rate of L-leucine efflux increases with decreasing H^+ concentration (9), with an apparent pK of about 6.8. This phenomenon can be explained in terms of an increase in the rate of deprotonation of the carrier on the outer surface of the membrane with decreasing external H^+ concentration. At low external pH, H^+ release will be slow, thereby limiting the rate of L-leucine efflux. Alternatively, the pH dependency of L-leucine efflux can be attributed to the degree of protonation of the carrier at the outer membrane surface, favoring the unprotonated form at more alkaline pH and assuming that only this form can recycle. The pH effect on L-leucine efflux is not easily explained by an internal pH effect since a low internal pH will favor H^+ binding to the carrier (increase the degree of protonation) on the inner surface of the membrane, which should enhance the rate of efflux if the scheme of the translocation process is correct (Fig. 3). Furthermore, in the presence of a saturating external concentration of L-leucine, L-leucine exit (exchange) is independent of the pH (Fig. 5B), suggesting that deprotonation of the carrier on the outer surface of the membrane or the return of the unloaded carrier to the inner surface of the membrane is a rate-determining step in the efflux process.

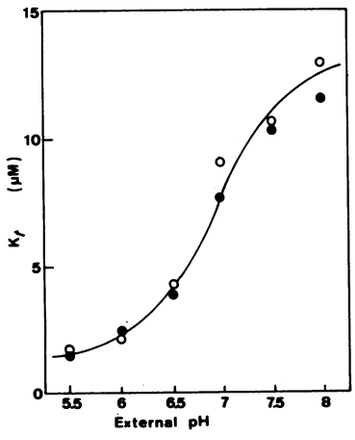

FIG. 4. Effect of external pH on K_t of L-leucine transport by membrane vesicles of *S. cremoris* Wg2 fused with asolectin liposomes (9). Transport was assayed in the presence of an imposed acetate diffusion gradient (ΔpH; ●) or a potassium diffusion gradient in the presence of valinomycin ($\Delta\psi$; ○).

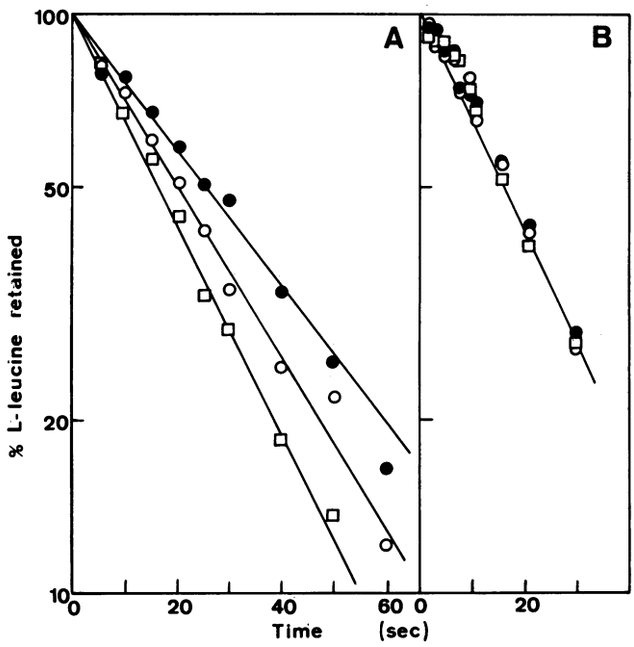

FIG. 5. Effect of pH on L-leucine efflux (A) and exchange (B) by membrane vesicles of *S. cremoris*. Membrane vesicles were equilibrated with 1 mM [^{14}C]leucine in 50 mM potassium phosphate buffer at pH 6.0 (●), 6.7 (○), and 7.5 (□). After 3 to 5 h of incubation at 25°C, samples (4 μl) were rapidly diluted into 50 mM potassium phosphate–5 mM $MgSO_4$ buffer of the pH indicated in the absence (A) and presence (B) of 1 mM [^{12}C]leucine.

In contrast to the K_t of L-leucine, the K_t values for L-alanine and L-serine transport were found to be essentially pH independent within the pH range 5.5 to 8.0 (12). The rates of L-alanine and L-serine efflux were shown to decrease with increasing pH, but this phenomenon does not appear to be caused by an external pH effect, as discussed below.

Recently, a novel cation antiporter in *S. lactis* has been described that catalyzes the stoichiometric exchange of L-arginine for L-ornithine (12a, 33). This transport system, which catalyzes the first step of the arginine deiminase pathway, has maximal activity at external pH values of 6.0 to 7.0; the V_{max} is about 50% reduced at pH 5.0 and 8.0. The K_t for L-arginine uptake appears to be independent of the external pH between 5.0 and 8.0.

Internal pH Effects

Internal pH effects on Δp-driven transport systems are usually much more difficult to recognize than external pH effects. The internal pH is determined by both the external pH and the magnitude of ΔpH. The ΔpH is one of the components of Δp which contributes to the driving force for the translocation of uncharged amino acids. This complicates a discrimination between effects of internal pH and the driving force (ΔpH) on transport. In this respect, the ionophore nigericin exerted some interesting effects on amino acid uptake by membrane vesicles of *S. cremoris* that have been fused with cytochrome *c* oxidase proteoliposomes (9,

11a, 12). In the fused membranes, in which cytochrome *c* oxidase is responsible for Δp generation, a compensatory increase of Δψ is observed when ΔpH is dissipated by nigericin. This phenomenon has been observed in the pH range 5.5 to 8.0. Thus, under conditions in which the magnitude of Δp remains the same, the magnitude of ΔpH can be varied. At pH 6.0, nigericin inhibits L-leucine uptake (Fig. 6A), while L-alanine uptake (Fig. 6B) and L-serine uptake are stimulated with respect that in the absence of this ionophore. At pH 6.0, the addition of nigericin results in a decrease of the V_{max} of leucine uptake from 0.8 to 0.4 nmol min^{-1} mg of $protein^{-1}$, while at pH 7.0 the V_{max} of leucine uptake is hardly affected by nigericin. V_{max} values of 1.7 and 1.6 nmol min^{-1} mg of $protein^{-1}$ are found at pH 7.0 in the absence and presence of nigericin, respectively. Also, the V_{max} of L-leucine uptake at pH 7.0 is significantly higher than at pH 6.0, despite a lower magnitude of Δp, i.e., −90 mV at pH 7.0 versus −110 mV at pH 6.0. In the absence of nigericin the internal pH values at pH 6.0 and 7.0 are 6.6 and 7.1, respectively. These data indicate that the rate of L-leucine uptake is affected by the internal pH. Lowering the internal pH by the addition of nigericin results in a decrease of the maximal rate of uptake. This effect is most likely explained by a catalytic effect of the internal pH on carrier function. Within the kinetic model of H^+/L-leucine cotransport depicted above, deprotonation of the carrier on the inner surface of the membrane is faster (thus the degree of protonation is lower) in the presence of a ΔpH (i.e., at higher internal pH) (9).

The effects of internal pH on transport of L-serine and L-alanine are completely different from its effects on L-leucine transport. The rates of L-serine and L-alanine efflux decrease with increasing pH (when the internal pH is equal to the external pH) (12); with a pK_a of about 7.0. A similar pH dependency is observed for four other modes of facilitated diffusion, i.e., influx driven by the L-serine or L-alanine gradient, Δp-driven uptake, exchange, and counterflow (11a, 12).

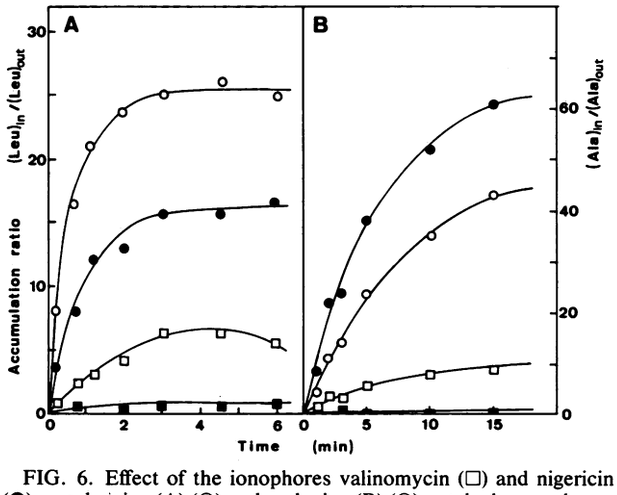

FIG. 6. Effect of the ionophores valinomycin (□) and nigericin (●) on L-leucine (A) (○) and L-alanine (B) (○) uptake by membrane vesicles of *S. cremoris* Wg2 fused with proteoliposomes containing cytochrome *c* oxidase at pH 6.0. Δp was generated by cytochrome *c* oxidase upon addition of the electrondonor system ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylene-diamine/cytochrome *c*. Nigericin and valinomycin were used at final concentrations of 10 and 100 nM, respectively. Transport in the absence of ascorbate is denoted by ■.

At high pH hardly any uptake of L-alanine and L-serine is observed, in contrast to the uptake of L-leucine. The Δp generated by cytochrome *c* oxidase in fused membranes at alkaline pH values is largely composed of Δψ. The ionophore valinomycin, which induces a collapse of Δψ with a partial compensating increase in ΔpH, completely inhibits L-alanine and L-serine uptake above pH 6.0. Uptake of these amino acids is only partially inhibited by valinomycin at or below pH 6.0. The inhibition of uptake is more severe than can be explained by the decrease in Δp. Furthermore, under conditions of full inhibition of L-alanine and L-serine transport by valinomycin, L-leucine uptake still occurs. The ionophore nigericin, which dissipates the ΔpH concomitant with a compensatory increase of the Δψ, stimulates the rate of L-alanine uptake (Fig. 6B). This stimulatory effect on both the rate of uptake and the final uptake level can be observed within the pH range 5.5 to 7.5, but the effect is most pronounced below pH 7 (Fig. 7, inset) (11a, 12). Similar observations have been made for L-serine uptake. The stimulatory effect of nigericin on L-alanine and L-serine uptake is in apparent conflict with the finding that Δψ (uptake in the presence of nigericin) and ΔpH (uptake in the presence of valinomycin) alone are able to drive uptake of these two amino acids (at least at pH 6.0 and lower).

The effects of valinomycin and nigericin on the uptake of L-alanine and L-serine can be satisfactorily explained only when the effects on the internal pH in addition to those on the Δp are considered. For this the rates of L-alanine uptake are measured in the presence and absence of nigericin and plotted as a function of the external and internal pHs (Fig. 7). As a function of the external pH, two different relations are observed (Fig. 7, inset). However, a unique relationship is found when the rate of L-alanine uptake is plotted as a function of the internal pH (Fig. 7). The internal pH in the presence of nigericin is equal to the external pH, whereas the internal pH without nigericin equals the external pH plus the ΔpH. At different pH values tested, the magnitude of Δp was the same in the presence and absence of nigericin. A similar relation is observed for L-serine uptake (Fig. 7). The pK_a estimated for the pH-sensitive group involved in this process is about 7.0.

The effect of internal pH on L-alanine and L-serine transport is exerted on the V_{max} of transport, whereas the K_t remains unchanged (12). If this pH effect represented a catalytic effect, one would expect that the rate of deprotonation of the carrier at the inner surface of the membrane would become faster with increasing internal pH. Since the opposite effect is observed, the internal pH most likely regulates these carriers allosterically.

The strong dependency of the L-alanine carrier on the internal pH leads to some unusual relationships between Δp and the uptake and efflux of the L-alanine analog α-aminoisobutyric acid (AIB) by intact cells of *S. lactis* (42). The initial rate of AIB uptake is inhibited by potassium ions, whereas potassium induces a rapid efflux of AIB under steady-state conditions of transport (Fig. 12 in reference 42). Under similar conditions, the initial rate of L-leucine uptake is stimulated by potassium ions (unpublished results). Addition of potassium ions to *S. lactis* cells causes a partial interconversion of the Δψ into a ΔpH without significantly affecting the magnitude of the Δp (B. Poolman, unpublished results). Inhibition of the initial rate of AIB uptake by potassium at constant driving force can be explained by the increase of the intracellular pH (Fig. 7). In accordance with

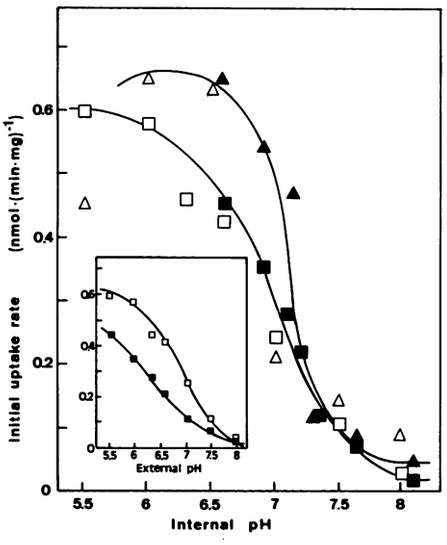

FIG. 7. Effect of internal pH on initial rate of uptake of L-serine (△, ▲) and L-alanine (□, ■) by membrane vesicles of *S. cremoris* Wg2 fused with cytochrome *c* oxidase proteoliposomes. Δp was generated as described in the legend to Fig. 6. In the absence of ionophores, the internal pH was calculated from the transmembrane pH gradient, measured with membrane vesicle-entrapped pyranine, and the external pH (closed symbols). In the presence of nigericin (10 nM), internal pH was equal to external pH (open symbols). (Inset) Effect of external pH on initial rate of L-alanine uptake in the absence (■) or presence (□) of 10 nM nigericin. Data were taken from reference 12.

the opposite internal pH dependency of the branched-chain amino acid carrier (see above), L-leucine uptake is expected to be stimulated by potassium ions. To explain the effect of potassium on the steady-state level of AIB, information is required about the pathways of entry and exit of AIB in addition to the internal pH dependency of the carrier. Under conditions of solute accumulation, the rate of inwardly directed AIB transport will be carrier mediated and dependent on the proton motive force. The rate of outwardly directed AIB transport is most likely composed of a carrier-mediated and a passive diffusion component (11a). Both fluxes are balanced under steady-state conditions of accumulation. If under steady-state conditions the AIB carrier is inactivated due to an increase of the internal pH (upon the addition of potassium ions), this will equally affect the rate of carrier-mediated influx and efflux. Passive efflux of AIB, however, will not be affected by a change in internal pH (11a). Upon an increase of the intracellular pH, the rate of AIB uptake is no longer sufficient to prevent efflux of AIB by passive diffusion until a new steady-state level is reached.

In the preceding sections on Δp-driven transport systems, the external and internal pHs have been shown to exert multiple effects on solute transport. These effects of pH can be opposite for transport systems with the same mechanism of energy coupling. The pH effects on L-leucine transport can be explained satisfactorily by the pH dependencies of partial reactions that occur in the translocation cycle of the branched-chain amino acid carrier. These pH effects have been designated catalytic. In contrast, the pH dependence of L-alanine and L-serine transport is the same for entry and exit as well as for exchange. The pH effects on L-alanine and L-serine transport do not likely represent rate-determining (de)protonation steps in the catalytic mechanism of the corresponding transport systems which means that these are allosteric effects.

Unspecified pH Effects

Multiple pH effects have also been reported for an anion antiporter of *S. lactis* 7962. This transport system catalyzes homologous exchange of phosphate and heterologous exchange of phosphate and glucose or mannose 6-phosphate (3, 32). Both the stoichiometry of the exchange process and the charge translocation will depend on the ionic forms of the substrates, i.e., phosphate (pK_2 = 7.2) and sugar phosphate (pK_2 = 6.1). The K_t values of homologous phosphate exchange in washed cells are constant between pH 5.5 and 6.5 and increase somewhat (i.e., from 200 to 330 μM) when the pH approaches the pK_2 of phosphate, indicating that monovalent phosphate is the preferred substrate (32). The maximal rate of phosphate exchange increases about 40-fold when the pH is raised from 5.5 to 7.2. Although sidedness of the pH effect is not specified, it can be suggested that protonation of a group with a pK_a of 6.5 to 7.0 determines the activity of the carrier. This pH effect can be designated an allosteric modification of the carrier protein.

Heterologous exchange studies have been performed with membrane vesicles loaded with either phosphate or 2-deoxyglucose 6-phosphate (4). The results suggest that the exchange stoichiometry depends on the pH of the medium. At pH 7.0 and above, 2 molecules of phosphate are translocated per molecule of sugar 6-phosphate, whereas at pH 5.2 this stoichiometry drops to 1 (4). The exchange reaction is electroneutral under all conditions tested. The K_t for the sugar 6-phosphate increases from 5.7 to 12.5 μM when the pH is increased from 5.2 to 7.0, i.e., 0.9 pH unit below and above the pK_2 of 2-deoxyglucose 6-phosphate, respectively. The slight change in affinity for the sugar 6-phosphate indicates a random choice among available mono- and divalent forms of this substrate. The pH dependence of the exchange stoichiometry can therefore be attributed entirely to the pH effect on the valence of the sugar 6-phosphate (4). Thus, the carrier catalyzes exchange of monovalent phosphate and monovalent sugar 6-phosphate in a 1:1 ratio at low pH, while at high pH, 2 molecules of monovalent phosphate are translocated per molecule of divalent sugar 6-phosphate. Asymmetry in the observed pH effect has not been found.

PHOSPHATE BOND-DRIVEN SOLUTE TRANSPORT SYSTEMS

Transport of several solutes, such as L-glutamate, L-glutamine, L-aspartate, L-asparagine, phosphate, and L-leucyl-L-leucine, by *S. lactis* and *S. cremoris* proceeds in the absence of a proton motive force (34, 36; unpublished results). With the exception of L-leucyl-L-leucine, which is hydrolyzed intracellularly, large concentration gradients of these solutes can be formed. Although the exact nature of the energy source has not yet been established, uptake of these metabolites requires the synthesis of ATP by either glycolysis or the arginine dihydrolase pathway (34, 36).

Despite the dependence on phosphate bond energy, variations in the magnitude of the ΔpH have been shown to be reflected directly in the activity of transport systems for L-glutamate and L-leucyl-L-leucine (34, 36). For instance, the rate of L-glutamate uptake and the magnitude of the ΔpH in *S. lactis* cells decrease in parallel with increasing pH of the medium (Fig. 8). Upon addition of the ionophore nigericin,

which abolishes the ΔpH, a complete inhibition of L-glutamate transport is observed in the pH range investigated. The apparent relationship between L-glutamate transport activity and ΔpH (Fig. 8, inset), however, can be explained entirely by an external pH effect on the concentration of the transported solute and an internal pH effect on the kinetic properties of the transport system (see below).

External pH Effects

Kinetic analysis of L-glutamate and L-glutamine uptake by *S. lactis* and *S. cremoris* has indicated that these amino acids compete for the same transport system (36). The affinity constant (K_t) for L-glutamate uptake at pH 6.0 appeared to be 30-fold higher than for L-glutamine (Fig. 9). Since dicarboxylic amino acids like glutamate and aspartate contain an additional protonizable group with pK_2 values of 4.25 and 3.86, respectively, different species dominate depending on the pH of the solution. To examine whether the protonated (glutamic acid) or the unprotonated (glutamate anion) species is transported, the dependence of the K_t of L-glutamate uptake on the external pH has been analyzed. The K_t of L-glutamate uptake (calculated from the sum of all glutamate species present in the solution) increased approximately 1 order of magnitude per unit increase in pH of the medium (Fig. 9). Calculation of the affinity constants on the basis of the concentrations of glutamic acid according to the Henderson-Hasselbach equation showed that the K_t is independent of the pH of the medium (Fig. 9, inset). Under identical conditions, the K_t for L-glutamine uptake was found to be independent of the extracellular pH (Fig. 9, inset), indicating that protonation of an ionizable group of the transport system is not responsible for the observed pH effect. From these experiments it is concluded that the external pH affects L-glutamate transport by changing the concentration of L-glutamic acid, the transported species. A

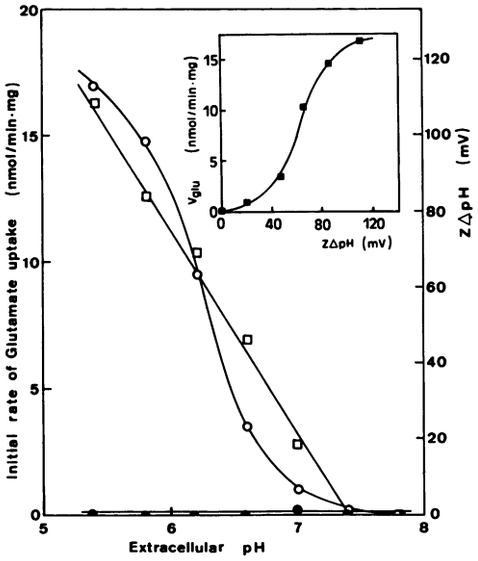

FIG. 8. Dependence of initial rate of L-glutamate uptake and magnitude of the pH on external pH in *S. lactis* ML3. Uptake of glutamate (100 μM, final concentration) was measured in the presence (●) and absence (○) of nigericin (0.63 nmol/mg of protein), with lactose as energy source. The magnitude of the ZΔpH (□) was determined from the distribution of salicylate, using an ion-selective electrode (36). ■, Apparent relationship between the initial rate of glutamate transport and ΔpH.

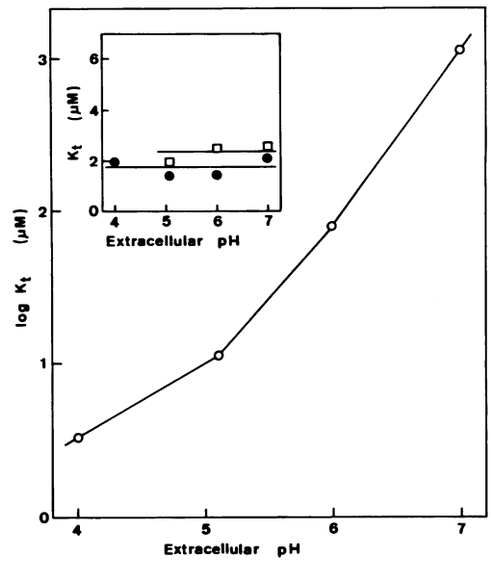

FIG. 9. External pH dependence of the affinity constant (K_t) for L-glutamate transport in *S. lactis* ML3 (36). The cells were energized by the addition of lactose. K_t values were calculated from the (total) concentration of all L-glutamate species present. (Inset) ●, External pH dependence of the K_t on the assumption that L-glutamic acid is the transported substrate; □, external pH dependence of the K_t for L-glutamine transport.

less detailed study of the accumulation of L-aspartate by *S. lactis* has indicated a similar external pH effect on the uptake of this amino acid.

The properties of the dicarboxylic amino acid transport system(s) in *S. faecalis* appear to differ from those in *S. lactis* and *S. cremoris*. Kinetic studies of the uptake of dicarboxylic amino acids by *S. faecalis* demonstrated competition between L-glutamate and L-aspartate for the same transport system (38, 43). The K_t values for L-glutamate and L-aspartate at pH 6.5 were 30 and 10 μM, respectively. Since at pH 6.2 and 7.5 the rates of L-aspartate uptake at a concentration of 10 μM (i.e., the sum of the acidic and anionic species) are very similar (17), it is unlikely that in *S. faecalis* L-aspartic acid (and L-glutamic acid) is the transported solute.

Another anion transport system in streptococci that has been analyzed with respect to the pH dependence of its kinetic properties is the phosphate translocator of *S. lactis* (35). The relevant species of phosphate present in solutions around neutral pH are the mono- and dianionic forms (pK_2 = 7.2). The K_t of phosphate uptake at pH 6.2, 7.2, and 8.2 is 6.5 ± 0.5 μM, indicating that the transport system has no preference for any of the phosphate ions.

These examples have shown the influence of the medium pH on the kinetics of solute transport by affecting the available concentration of the transported solute. The importance of the elucidation of these kinetic properties with respect to growth of lactic acid streptococci is discussed below. Direct effects of the medium pH on the K_t of the transport protein have also been observed. In *S. cremoris* the affinity constant for L-leucyl-L-leucine transport increases with increasing pH. The K_t values for L-leucyl-L-leucine uptake are 32, 185, and 450 μM at pH 5.2, 6.2, and 7.4, respectively (44). Since L-leucyl-L-leucine is neutral in the pH range investigated, these results indicate that the external pH directly alters the affinity of the transport

system. This external pH effect is probably allosteric, since vectorial proton movement does not appear to occur in the translocation of L-leucyl-L-leucine.

Internal pH Effects

Transport of L-glutamate/L-glutamine and phosphate by *S. lactis* proceeds in the absence of a proton motive force as already pointed out above. The addition of protonophores or ionophores, which dissipate the pH gradient across the cytoplasmic membrane, to cells metabolizing lactose or arginine can, however, result in a severe inhibition of transport depending on the pH of the medium. These apparently contradictory results can be explained by taking into account the internal pH dependence of these transport systems.

The dependencies of the initial rates of L-glutamate and phosphate transport on the intracellular pH are shown in Fig. 10. The pK_a values are 7.0 and 7.3 for the uptake of L-glutamate and phosphate, respectively. For L-glutamate transport, the cells were energized by the addition of lactose, whereas the internal pH was varied by the addition of nigericin at both pH 5.0 and 6.0 or by the amount of potassium added in the presence of valinomycin. Phosphate transport was measured at various external pH values in the presence of an excess of nigericin and potassium ions. The corresponding intracellular pH values were taken from an experiment similar to that shown in Fig. 2. To prevent metabolism of accumulated inorganic phosphate, *S. lactis* cells have been energized by L-arginine as the source of ATP synthesis (35).

Preliminary experiments on the phosphate bond-dependent transport systems for L-aspartate and L-leucyl-L-leucine indicate a similar internal pH dependence for these transport systems. The requirement of a neutral or slightly alkaline cytoplasm has also been observed for maximal activity of arsenate and phosphate transport in glycolyzing cells of *S. faecalis* (17). The internal pH dependence of arsenate transport titrates with a pK_a of about 7.2. Also, the ATP-driven exchange of Na^+ and K^+ ions by *S. faecalis* appears to be regulated by the intracellular pH (18, 22). Sodium extrusion by this system, previously designated as the ATP-driven sodium pump in *S. faecalis* (19), is not affected by valinomycin plus tetrachlorosalicylanilide at pH 7.4 (or above), but is slowed down somewhat at pH 7.0 and blocked completely at pH 6.6 (or below) (19).

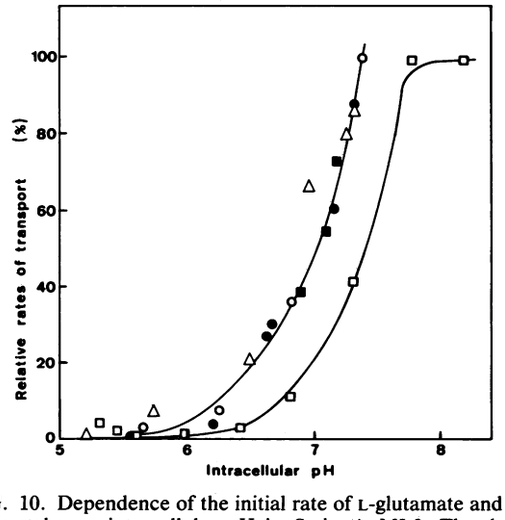

FIG. 10. Dependence of the initial rate of L-glutamate and phosphate uptake on intracellular pH in *S. lactis* ML3. The data for L-glutamate transport (○, ●, ■, △) were taken from reference 34. The internal pH was varied by the amount of nigericin in the medium at both pH 5.0 (○) and 6.0 (●, ■) or by the amount of potassium added in the presence of valinomycin at pH 5.8 (△). For phosphate transport (□), the internal pH was set by varying the external pH in the presence of nigericin (35). For the uptake of L-glutamate and phosphate, the cells were energized by the addition of lactose and L-arginine, respectively.

The regulatory intracellular pH effects on the phosphate bond-driven transport systems can be classified as allosteric, since protons are not (directly) involved in the energy-coupling mechanism (see above) (34, 36). Similar to the relation between L-alanine (and L-leucine) uptake and the (cat)ionic composition of the medium (see above), the dependencies of the phosphate bond-driven transport systems on potassium can be explained entirely by effects on the intracellular pH (34). In contrast to the Δp-driven transport systems, the phosphate bond-driven transport systems studied thus far all function optimal at slightly alkaline pH values.

PHYSIOLOGICAL SIGNIFICANCE OF pH EFFECTS ON SOLUTE TRANSPORT FOR GROWTH

Growth of *S. cremoris* and *S. lactis* (group N streptococci) is generally found to be restricted to an upper pH value of about 7 (21; R. Otto, Ph.D. thesis, University of Groningen, Haren, The Netherlands, 1981). In contrast, *S. faecalis* (group D streptococcus) exhibits a growth rate maximum that ranges from pH 6 to 8.5 (23). For both groups of organisms growth starts to decline below pH 6. This effect appears to be most pronounced in media with high concentrations of weak acids (21; Otto, Ph.D. thesis), which affects the maintenance of the intracellular pH (41).

Since *S. cremoris* and *S. lactis* can regulate their internal pH between 7.0 and 7.5 at external pHs of 5.5 to 7.5 (Fig. 2) (41), the failure to grow at alkaline pH is unlikely to be caused by a limitation of cytoplasmic processes. In the preceding section in which the external pH effects on L-glutamate transport are discussed, it was shown that *S. lactis* and *S. cremoris* accept and transport only the protonated form of the amino acid (Fig. 9). Since these streptococci can synthesize L-glutamate only from L-glutamine, glutamate (or glutamine) is essential for growth. At around neutral and alkaline pH the dominant L-glutamate species is the anion. It can be calculated from the amount of L-glutamate required for biosynthetic purposes that the maximal growth rate of *S. lactis* and *S. cremoris* above pH 7 is limited by the capacity to accumulate L-glutamate (B. Poolman and W. N. Konings, submitted for publication). Even with millimolar concentrations of glutamate in the medium, the glutamic acid concentrations at alkaline pH may become lower than the K_t for uptake. On the other hand, with L-glutamine present in the growth medium, the growth rate of *S. lactis* (and *S. cremoris*), unrestricted by the supply of glutamate, proceeds at a high rate up to pH 8 (Fig. 11). The accumulation of L-aspartate by *S. lactis* and *S. cremoris* is also restricted at alkaline pH because only the acidic form is transported. However, L-aspartate, in contrast to L-glutamate, is not an essential amino acid for these bacteria (30).

Based on the differences in the kinetics of L-glutamate uptake by *S. lactis* (and *S. cremoris*) and *S. faecalis*, it is possible to understand why the latter organism can grow at alkaline pH in the presence of L-glutamate (23). The *S.*

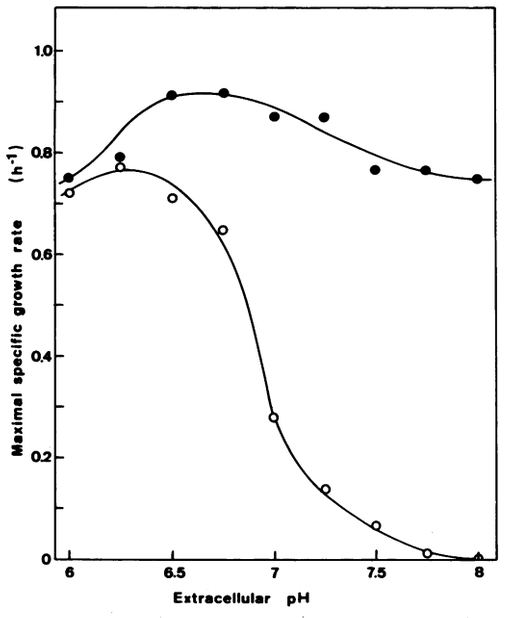

FIG. 11. pH dependence of growth of *S. lactis* ML3. *S. lactis* was grown in a chemically defined medium at 30°C with 2.67 mM L-glutamate (○) or L-glutamine (●) as sources of L-glutamate and L-glutamine for biosynthesis. Further details of the growth medium have been described in Otto (Ph.D. thesis), with the exception that the potassium phosphate concentration was increased to 105 nM. Growth was measured up to a final optical density at 660 nm of 0.3 (Poolman and Konings, submitted).

faecalis transport system has no preference for either the acidic or the anionic form of L-glutamate, and even when the organism only transports the L-glutamate anion, the dominant species at alkaline pH, uptake of L-glutamate will not become a limiting factor for growth at pH 6.0 and 8.5.

As already mentioned above, the lower pH limits for growth of streptococci are related to the capacity to alkalinize the cytoplasm (23, 41). The sharp decline in activity of the phosphate bond-dependent transport systems when the internal pH falls below 7.5 (Fig. 10) can be a primary reason for growth inhibition at acidic pH values. In fact, it has been observed that growth of *S. cremoris* is less sensitive to the uncoupler SF6847 in media containing a tryptic casein digest than in media containing amino acids only (Otto, Ph.D. thesis). In the presence of the casein digest, the organism will have an additional capacity to accumulate essential amino acids since these nutrients can also be taken up as peptides of varying chain lengths by the corresponding peptide transport system(s).

CONCLUDING REMARKS

The major purpose of this review was to analyze the various regulatory pH effects on solute transport in lactic streptococci. As exemplified by the pH effects on L-glutamate transport in *S. lactis*, only a full kinetic analysis of the pH dependence of transport activity can distinguish between regulatory pH effects and the involvement of ΔpH as a driving force.

The internal pH of bacteria (and to some extent of membrane vesicles) is largely determined by the ionic composition of the medium (8). Effects of (cat)ions on solute transport can therefore be secondary, i.e., due to changes in the internal pH. The interrelation between the effect of cations on amino acid transport and the internal pH have been demonstrated clearly for a number of transport systems.

Regulatory pH effects on solute transport in microorganisms have thus far hardly been recognized. The only exceptions are those (cation) transport systems thought to be involved in pH homeostasis in bacteria (6, 7). In contrast, control of transport processes by pH changes in mammalian cell types are relatively well documented (15). The internal and external pH effects on solute transport summarized in this paper all occur within the physiological pH range (Table 1), which stresses the important role of pH in regulating these processes. In contrast to regulation by the intracellular pH of cation transport systems, which in turn may regulate the cytoplasmic pH, the purpose of regulating amino acid transport systems by the intracellular pH is not directly clear. With the exception of the carriers for L-alanine and L-serine in *S. cremoris*, the activity of most solute transport systems increases with increasing internal pH and maximum activity is reached between pH 7.5 and 8.0. It can be calculated that the amount of metabolic energy required in lactic acid streptococci to accumulate at maximal rates individual solutes such as L-glutamate (L-glutamine), L-asparagine, phosphate, L-leucine, and others can be up to 10% of the rate of metabolic energy (ATP) generation by glycolysis. The lower activities of these transport systems at acidic internal pH values, when the activities of the glycolytic pathway and other metabolic processes are also low, may serve to protect the organisms from wasting metabolic energy by "unbalanced" uptake. Under energy-sufficient conditions, most, if not all, neutrophilic bacteria will maintain an internal pH between 7 and 8. Only when energy

TABLE 1. Effect of external and internal pH on solute transport in *S. lactis* ML3 and *S. cremoris* Wg2

Transport	Energy-coupling mechanism	External pH			Internal pH		
		Kinetic parameter	Type of effect	pK_a[a]	Kinetic parameter	Type of effect	pK_a[a]
Glu/Gln	~p	K_t^{Glu}	Substrate*	4.25*	V_{max}	Allosteric	7.0
Asp	~p	K_t^{Asp}	Substrate*	3.86*	V_{max}	Allosteric	?
Leu-Leu	~p	K_t^{uptake}	Allosteric	?	V_{max}	Allosteric	?
Phosphate	~p	None		7.20*	V_{max}	Allosteric	7.2
Leu/Ile/Val	Δp	K_t^{uptake}	Catalytic	7.0	V_{max}	Catalytic	7.0
		V_{max}^{efflux}	Catalytic	6.8			
Ala/Gly	Δp	None			V_{max}	Allosteric	7.0
Ser/Thr	Δp	None			V_{max}	Allosteric	7.0

[a] pK_a refers to the pH dependence of the transport system except when an asterisk is present, which signifies that the pH effect is due to a change in the available substrate concentration as a result of changes in the relative concentration of the protonated species; the corresponding pK_a values of the substrates are given.

becomes limited will the internal pH decrease. Under those conditions the organism will focus primarily on strategies for survival, which include reducing the metabolic energy consumption for uptake of solutes and preventing exit of metabolites. Both effects are achieved by a decreased activity of the transport processes at decreased internal pH values.

The internal pH effects on solute transport discussed in this paper have all been observed in streptococci. The relevance of these regulatory pH effects is further strengthened by observations made in other bacteria. For example, the activity of the alanine carrier of *Rhodobacter sphaeroides* and the lactose carrier of *Escherichia coli*, which has been cloned and expressed functionally in cells of *R. sphaeroides*, increase with increasing internal pH at constant values of Δp (T. Abee, unpublished results). The pK_a values estimated from these titration curves are 7.8 and 7.2 for the alanine and lactose carriers, respectively. In addition to these data, it is often observed that ionophores inhibit solute transport in bacteria more effectively at acidic pH than at alkaline pH. For instance, the pH dependence of the plasmid-encoded arsenate efflux system of *E. coli* and *Staphylococcus aureus* in the presence and absence of ionophores indicates that this system requires an alkaline intracellular pH for maximal activity (40). We believe that the various regulatory pH effects on solute transport in streptococci may not be restricted to these bacteria, but rather that this type of regulation is widespread among microorganisms.

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